CHROMATOGRAPHIC SEPARATION OF PHOSVITIN, α- AND β-LIPOVITELLIN OF EGG YOLK GRANULES ON TEAE-CELLULOSE[1]

M. W. RADOMSKI[2] AND W. H. COOK
Division of Biosciences, National Research Council, Ottawa, Canada

Received April 16, 1964

Abstract

The two components of lipovitellin and the three major components of yolk granules, phosvitin, α- and β-lipovitellin, have been separated by gradient elution chromatography on TEAE-cellulose. A 0.2 *M* phosphate buffer (pH 6.8) had the necessary ionic strength to dissolve these proteins and when applied in this solvent all components except β-lipovitellin were retained by the column. A linear gradient of ionic strength (limit buffer 0.2 *M* phosphate plus 0.5 *M* NaCl) was used to remove the other components. Recovery was essentially complete and the composition and properties of the individual components were similar to those obtained by previous chromatographic methods that gave only partial recovery. An additional component eluted after α-lipovitellin and before phosvitin, previously observed in Dowex-1 separations, was also observed by the present method. The composition, sedimentation behavior, and absorption spectra of this component indicate that it is a soluble complex of phosvitin and lipovitellin. When granules are dissolved in alkaline solvents (pH 9.4) of low ionic strength (0.05), phosvitin is not evident as a separate component during ultracentrifugation, but appears as the ionic strength is increased.

Introduction

The sedimenting granules in egg yolk have been shown (1, 2, 3) to contain the phosvitin (Pv) and lipovitellin (Lv). When the granules are dissolved, the Pv can be removed by chromatography on Dowex-1 and the Lv separated into two fractions (α-Lv and β-Lv) by chromatography on hydroxyapatite (3). The Lv fractions differ in their protein phosphorus content (α-Lv > 0.5% β-Lv ca. 0.3%), electrophoretic mobility (2), affinity for hydroxyapatite, and dissociation behavior (3), and these distinguishing features are evidently interrelated (4). Isolated β-Lv appears to be relatively homogeneous but α-Lv is a mixture of closely related molecular species of graded protein phosphorus content (4).

Essentially complete recovery of the granule proteins is desirable to insure that no major components are lost during isolation. The recovery of Pv from Dowex-1 columns was only 40–50% (3) and that of Lv from hydroxyapatite was only 60–70% (4). Hydroxyapatite also has the disadvantages of being unsuitable for large columns, and, because of limited stability, requires preparation at frequent intervals (3, 4, 5). Obviously, it would be desirable to have a phosphate-free system for separating materials that are characterized by their phosphorus content, although current evidence indicates that inorganic phosphorus can be removed completely from these proteins (3, 4).

Initially this study was undertaken to find an ion-exchange system that would

[1]Issued as N.R.C. No. 7991.

[2]Postdoctoral Fellow, National Research Council, Ottawa, Canada, 1961–1963. Present address: Defence Research Medical Laboratories, Toronto 12, Ontario.

separate the Lv fractions, give essentially complete recovery, and preferably avoid the use of phosphates. When most of these objectives were met by the use of triethylaminoethyl-cellulose (TEAE-cellulose), this new technique was applied not only to Lv but also to solutions of the granule proteins containing both Lv and Pv. This paper describes the technique used to separate these components, their identification and characterization, and some additional observations on the PvLv complex found in this and earlier studies (4).

Preparations and Methods

Granules were separated by the method described by Burley and Cook (3), thoroughly washed with water, dissolved and centrifuged to remove low density material. For preparing Lv the granules were dissolved in 0.7 *M* acetate buffer and after centrifugation were applied to Dowex-1 columns to remove Pv (3). These columns were twice as long (2.5 × 100 cm) as those used by Burley and Cook and would remove Pv from 22.7 g of granule solids before regeneration was necessary. The protein phosphorus content of the Lv obtained was 0.50 to 0.59%. These Lv solutions in acetate buffer were then dialyzed exhaustively against 0.2 *M* phosphate buffer pH 6.8 for application to the TEAE-cellulose columns. The limited solubility of Lv at 5° C in this solvent required the use of large initial volumes. For the preparative separations reported here 4.5 to 5 g of Lv were dissolved in a minimum of 200 ml of phosphate buffer.

Solutions of the granule proteins, containing Lv and Pv, were prepared in the same way except that they were dissolved directly in 0.2 *M* phosphate buffer. After centrifuging it to remove low density material, the solution was again dialyzed against several volumes of the same buffer before application to the TEAE-cellulose columns at the same loading as for Lv.

The procedure described by Peterson and Chiazze (6) was used as a guide in preparing the TEAE-cellulose (Bio-Rad, 0.5 meq/g) for chromatography. After washing with alkali and removing the "fines", the absorbent was suspended in the starting buffer, 0.2 *M* potassium phosphate pH 6.8 (3), and adjusted to this pH with the acidic buffer component. Preliminary experiments were performed on small columns (2.5 × 27 cm), but the results reported here were all obtained on larger (5 × 45 cm) preparative columns. Columns were packed at room temperature, transferred to a room at 5° C, and thoroughly washed with cold buffer before use.

Following application of the protein solutions the TEAE-columns were washed exhaustively with the starting buffer to remove the component that was not retained by this absorbent. The other components were then eluted from the columns with a linear gradient of increasing ionic strength obtained by using 1 l. of starting buffer (0.2 *M* phosphate in the mixing flask and 1 l. of limit buffer (0.2 *M* phosphate in 0.5 *M* NaCl) in the reservoir, the flask and reservoir being of the same dimensions. Conductivity measurements on the effluent were used to determine the gradient obtained. Flow rates of 35–45 ml/hr were used, effluent was collected in 10-ml aliquots, and the course of elution followed by measurements of optical density at 280 mμ.

The protein-containing effluents were pooled, dialyzed against 0.5 *M* NaCl, concentrated by pervaporation, and again dialyzed exhaustively against several changes of 0.5 *M* NaCl to remove inorganic phosphorus. Analysis of the dialyzed effluents for inorganic phosphorus gave negative results and as no reduction in the protein phosphorus of the Lv fractions occurred on passage through a Dowex-1 column (3), this step was omitted for some of the samples. Delipidation prior to analysis would also reduce or remove any traces of inorganic phosphorus.

Total lipid was determined by the method of Bligh and Dyer (7) with minor modification. The chloroform phase was either rewashed with water to remove traces of salts or dried and reextracted with ethyl ether. Similar results were obtained by both techniques.

The tyrosine–tryptophan ratio was obtained from the ultraviolet absorption (8) of solutions of delipidated protein (0.35 mg/ml) in 0.10 *N* NaOH, using a Cary Recording Spectrophotometer.

Other chemical and physical procedures have been described in previous communications (2, 4).

Results

Fractionation of Lipovitellin

Initial experiments were performed with Lv from which Pv had been removed by passing solutions of the granule proteins through Dowex-1. Columns of carboxymethyl cellulose at pH 6.8 did not retain Lv from solvents of the ionic strength required to dissolve Lv (0.3 μ). Columns of DEAE- and TEAE-cellulose likewise did not retain these proteins when applied in acetate buffer at pH 6.4 and 7.8, in veronal–HCl at pH 6.8, and in tris–HCl at pH 7.0. Of the neutral solvents tested, 0.2 *M* phosphate buffer at pH 6.8 was the only one that dissolved Lv and gave appreciable absorption on DEAE- and TEAE-cellulose. While both these ion-exchangers gave the same type of separation, TEAE appeared to give better resolution and was chosen for further work.

A typical elution pattern of Lv on the large TEAE-cellulose column is shown in Fig. 1. Fraction A is the Lv component that was not absorbed under the conditions employed and was washed out with additional starting buffer. Application of an essentially linear gradient of increasing ionic strength (dashed line) displaced fractions B, C, and D. Retention of fractions B and C was sensitive to small changes in ionic strength. Occasionally a small shoulder preceded fraction B, as indicated by the broken line in Fig. 1. On isolation and analysis this fraction showed the same composition as fraction A. With this exception all fractions appeared to be homogeneous and emerged at the same position when rechromatographed by the same procedure.

The proportions of the individual fractions and the total recovery of Lv estimated from optical density at 280 mμ are given in Table I. Recovery was essentially complete (97%), fraction A representing 55% of the total, B and C 36%, and the remainder (fraction D) about 10%.

Analytical ultracentrifugation of the fractions in 1 *M* NaCl yielded the patterns shown in Fig. 2. Fractions A, B, and C had a main component with

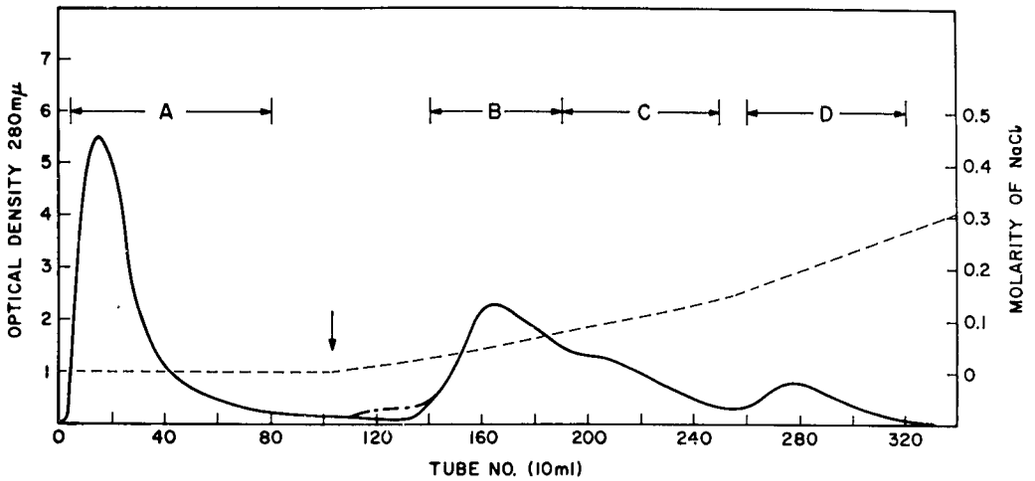


FIG. 1. Chromatography of lipovitellin solutions on a large TEAE-cellulose column. Starting buffer, 0.2 *M* phosphate pH 6.8; limit buffer, 0.2 *M* phosphate in 0.5 *M* NaCl. Start and course of gradient shown by vertical arrow and dashed line. Letters indicate fractions taken for anaylsis reported in Figs. 2 and 3 and Tables I and II. See text for further details.

TABLE I

Recoveries of Lv fractions from four fractionations on TEAE-cellulose columns

Fraction	(1), %	(2), %	(3), %	(4), %	Average, %
A	57	58	51	54	55
B, C	34	36	39	35	36
D	13	6	10	11	10
Total recovery	96	103	94	95	97

a sedimentation coefficient similar to that of α-Lv and β-Lv (i.e., 10.9 S). Fraction A also contained a slow minor component suggestive of monomer Lv that was not removed by rechromatographing. This may indicate that some dissociation of β-Lv occurs in this solvent at pH 6.8. A small amount of material sedimenting ahead of the main peak in fractions B and C may be the X-component that has been observed (4) in fractions of high protein phosphorus content. If this is a degradation (oxidized) product, it appears that oxidation and phosphorus content are related. Fraction D contained two components with sedimentation coefficients somewhat higher than those of monomer and dimer Lv.

Sedimentation in carbonate buffer at pH 9.0 and 0.2 μ showed that fraction A was largely dissociated into monomer while B and C showed little dissociation, a behavior characteristic of β-Lv and α-Lv respectively (3). Fraction D showed little change even at pH 10.6.

Chemical analyses of these fractions are given in Table II, together with those previously reported for α-Lv and β-Lv fractionated on hydroxyapatite. Comparison shows that the protein phosphorus content of fraction A and β-Lv,

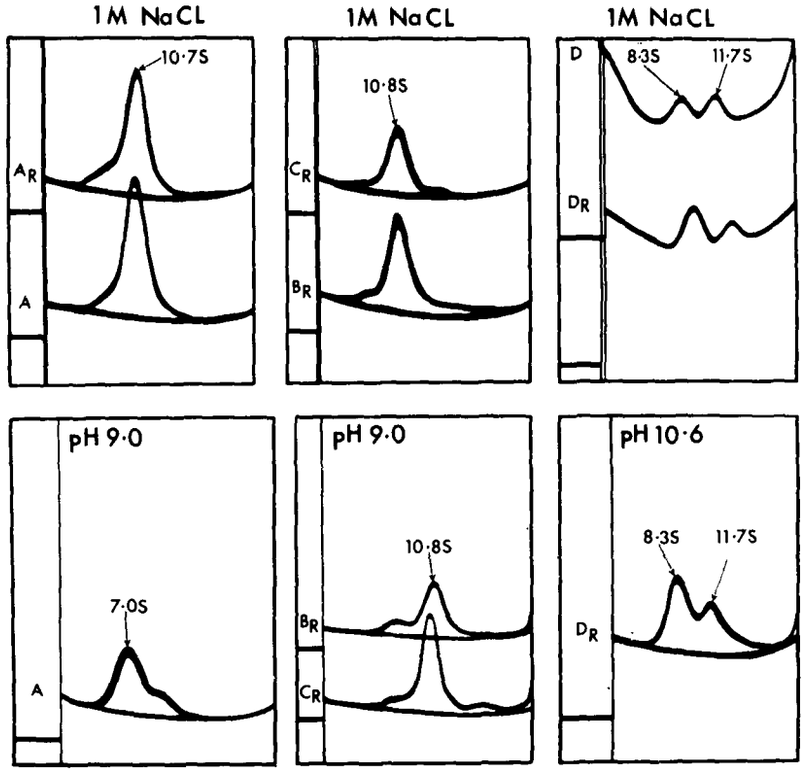


FIG. 2. Sedimentation patterns of fractions A to D shown in Fig. 1. Subscript R signifies rechromatographed sample. Solvents as indicated with carbonate buffer μ 0.2 used to obtain pH values in lower row. Peaks identified by $s^0_{20,w}$ value but time and speed of centrifugation variable.

TABLE II

Chemical analyses of lipovitellin fractions shown in Fig. 1

Constituent	Fraction and identification A β-Lv (%)	B α_1-Lv (%)	C α_2-Lv (%)	D PvLv complex (%)	Published results β-Lv, — (%)	α-Lv, — (%)	Ref.
Total nitrogen	13.3	13.4	13.5	14.0	—	—	
Total phosphorus	0.72	0.89	0.97	2.22	0.65	0.91	(3)
Lipid	16.5	14.6	13.5	—	21.7	22.2	(3)
Nitrogen in lipid	1.15	1.18	1.21	—	—	—	
Phosphorus in lipid	2.52	2.58	2.70	—	2.10	2.10	(3)
Phospholipid in lipoprotein (lipid P × 25)	10.4	9.4	9.1	—	11.6	11.6	(3)
Protein	83.5	85.4	86.5	—	78.3	77.8	(3)
Nitrogen in protein	15.2	15.0	15.1	14.2	15.8	15.7	(15)
Phosphorus in protein	0.28	0.54	0.69	1.65	0.27–0.35	0.50–0.73	(4)
Tyrosine/tryptophan ratio	2.65	2.65	2.40	1.23	—	—	
Dissociation (pH 9.0)	81	18	10	—	80	20	*

*Unpublished observations.

and fractions B and C and α-Lv, are similar. The protein phosphorus content (1.65%) of fraction D resembles the fraction isolated earlier from Dowex-1 containing 2.2% protein phosphorus (4) and interpreted as a PvLv complex. The lower protein nitrogen and lower tyr/try ratio of fraction D support the contention that Pv is present in this fraction.

Ultraviolet spectra of fractions A, B, C, and D are given in Fig. 3, together with those for Pv and the washed granule fraction for comparison. Absolute values for optical density have been omitted to permit vertical positioning of the curves to facilitate comparison. All curves represent identical protein concentrations on a dry weight basis. Spectra of fractions A, B, C, and those from α-Lv and β-Lv prepared from hydroxyapatite (unpublished observations) were indistinguishable. Fraction D differs from the other fractions and resembles that of the washed granules. Addition of Pv to Lv would raise the tyr maximum, and indicates that fraction D contains Pv and Lv in similar proportion to that found in washed granules. It seems unlikely that fraction D represents an entirely different protein since all its known properties are consistent with those of a PvLv complex.

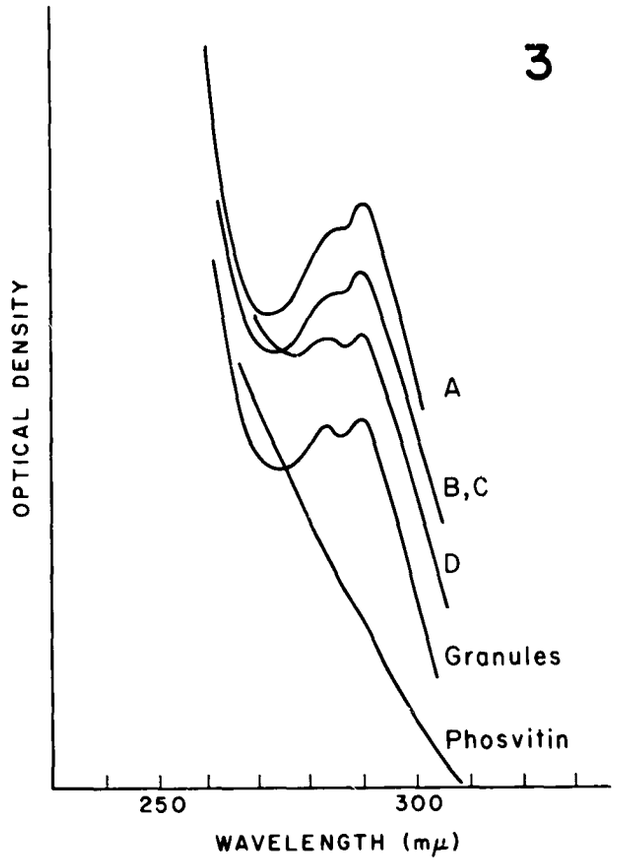
From the observed sedimentation coefficients, dissociation behavior, and protein phosphorus contents, it is evident that fraction A is β-Lv, fractions B and C are α-Lv (or α_1-Lv and α_2-Lv), and fraction D resembles the PvLv complex observed in earlier fractionations (4) performed on Dowex-1 and hydroxyapatite. Compared with previously reported values (3) these α- and β-Lv's have similar phospholipid – neutral lipid ratios but somewhat lower total lipid contents. Within these limits lipid content is not a reliable criterion for identification of the fractions, and the composition aspects will be discussed later.

Fractionation of Granule Proteins

The above results encouraged us to apply the procedure to solutions of the yolk granules without prior passage through Dowex-1 to remove Pv. Clearly, a higher recovery of all the granule proteins would permit a more complete description of their composition. Chromatographic conditions were the same as those used for Lv.

The elution pattern obtained is shown in Fig. 4. Fractions *a*, *b*, *c*, and *d* closely resemble their counterparts in Fig. 1, and these are followed by an additional component that was recovered as fractions *e* and *f*. Total recovery of the granule proteins, estimated from optical density, was again complete (102%) and the proportion of each of the fractions is given in Table III. Overall recovery of total phosphorus was also 102%.

Sedimentation patterns for these fractions are shown in Fig. 5. Those for *a*, *b*, and *c* in 0.5 *M* NaCl are similar to the corresponding fractions in Fig. 2; both fractions *a* (β-Lv) and *b* (mainly α-Lv) show some slower sedimenting component and the faster sedimenting X component is evident in both α-Lv fractions *b* and *c*. Fraction *d* shows the presence of two components sedimenting



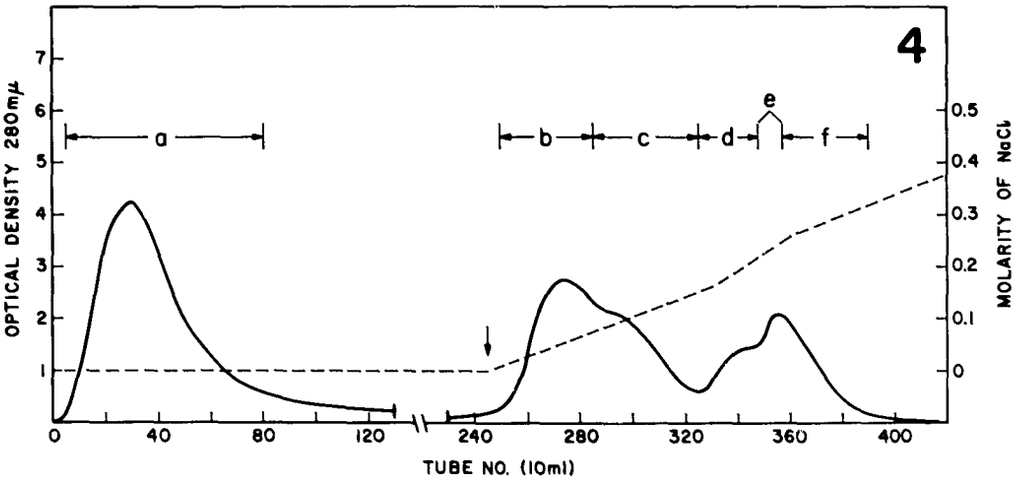


FIG. 3. Ultraviolet spectra of delipidated granule proteins, and fractions A to D shown in Fig. 1. See text for details.

FIG. 4. Chromatography of solutions of washed granule on a large TEAE-cellulose column. Solvents as in Fig. 1. Letters indicate fractions taken for analyses reported in Figs. 5 and 6 and Table III. See text for details.

TABLE III

Chemical analyses and recoveries of granule fractions shown in Fig. 4

Constituent	Fraction and identification					
	a	*b*	*c*	*d*	*e*	*f*
	β-Lv (%)	α_1-Lv (%)	α_2-Lv (%)	PvLv complex (%)	Mixture Pv and PvLv complex (%)	Pv (%)
Material recovered	43.3	29.0		5.6	6.2	15.9
Total nitrogen	13.3	13.4	13.3	13.9	12.6	11.5
Total phosphorus	0.73	1.02	1.10	2.30	5.37	8.63
Lipid	16.3	14.2	13.4	14.0	11.2	1.4
Protein	83.7	85.8	86.6	86.0	88.8	98.6
Nitrogen in protein	16.2	15.7	15.8	15.0	12.9	11.6
Phosphorus in protein	0.36	0.56	0.73	1.51	5.10	8.76
Tyrosine/tryptophan ratio	2.4	2.4	2.2	1.6	1.1	—

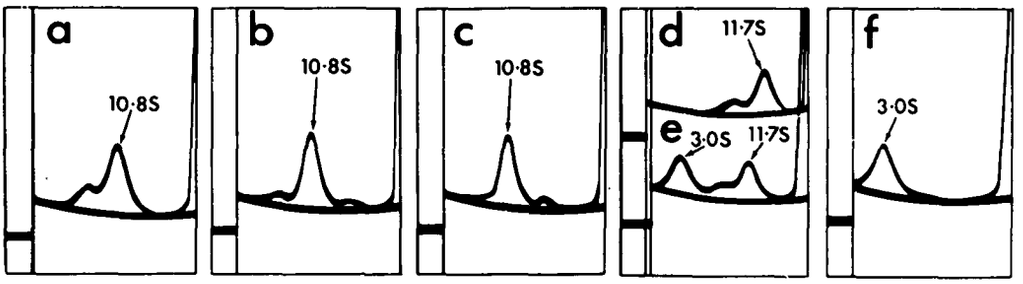


FIG. 5. Sedimentation patterns of fractions *a* to *f* as shown in Fig. 4. Solvent 0.5 *M* NaCl. Peaks identified by $s^0_{20,w}$ values, speed 42,040 r.p.m. but time variable.

at rates corresponding to those of D (Fig. 2) but the proportion of the slow component, containing the presumptive Lv monomer, is lower, a difference that may be attributable to the arbitrary eluant concentration ranges chosen. Fraction *e* taken at the maximum optical density of the last component is evidently a mixture of three components, one having the sedimentation rate of phosvitin (ca. 3.0 S) and the other two resembling those in fraction *d*. Fraction *f* is predominantly the slow-sedimenting Pv component.

Results of chemical analyses are given in Table III. The protein nitrogens of these fractions are higher than those of the corresponding fractions isolated from Lv but are comparable with published results given in Table II. Protein phosphorus in these fractions is also somewhat higher than in Lv (Table II) and may indicate the presence of trace amounts of Pv. This view is supported by the lower tyr/try ratio of the granule fractions since the presence of Pv would lower this ratio. Nevertheless, the values are within the range observed for α-Lv and β-Lv prepared by chromatographing on Dowex-1 and hydroxyapatite. Fraction *a*, eluted with the starting buffer, is evidently β-Lv, and fractions *b* and *c* the α-Lv's. Their lipid contents, while lower than reported

values (3), are comparable with those of fractions A, B, and C (Table II). Fractions *d* and D have comparable protein phosphorus contents and evidently both contain the presumptive PvLv complex (4). Fraction *e* apparently contains about 40% Pv in addition to fraction *d*, while fraction *f* is predominantly Pv.

The ultraviolet spectra of these fractions are shown in Fig. 6. Curves for fractions *a* to *c* are the same. Those for fractions *d*, D, and washed granules are also similar. The effect of increasing proportions of Pv is clearly shown by the transition of the curves from fractions *a* to *c*, which contain little or no Pv, through the several fractions to *f* that contains only Pv. This adds support to the view that fractions *d* and D contain a PvLv complex.

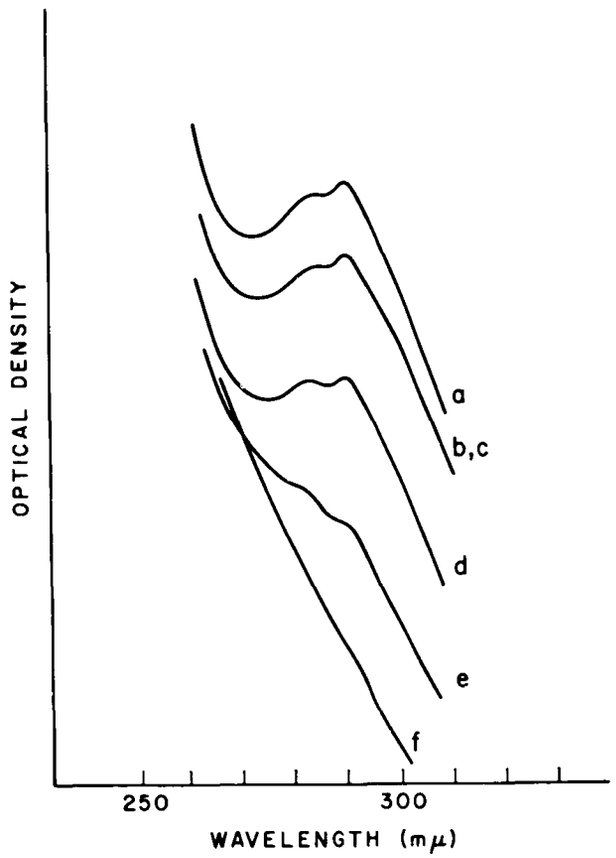


FIG. 6. Ultraviolet spectra of delipidated protein fractions *a* to *f* shown in Fig. 4. See text for details.

Effect of Ionic Strength of Sedimentation Behavior

The presence of components D and *d* indicates that the granules contain a component that is neither Pv nor Lv but may be a complex of both. Such a complex may represent the state of these proteins in the undissolved granule, and some supplementary experiments were undertaken to obtain more direct

evidence on this point. An ionic strength of 0.3 or higher is required to dissolve the granules in the region of neutrality, and this may break up the complex either directly or by replacing divalent ions (e.g., calcium) responsible for complex formation. Dissolution at low ionic strength (0.05) required the use of a buffer of high pH (carbonate buffer pH 9.4). To avoid the loss of exchangeable divalent ions, the granule solutions were not subjected to dialysis or other manipulations before examination in an analytical ultracentrifuge.

The patterns obtained from the granule proteins dissolved in carbonate buffer at pH 9.4 with ionic strengths ranging from 0.05 (buffer only) to 0.90 by NaCl additions are shown in Fig. 7. At this pH Lv will be partly dissociated

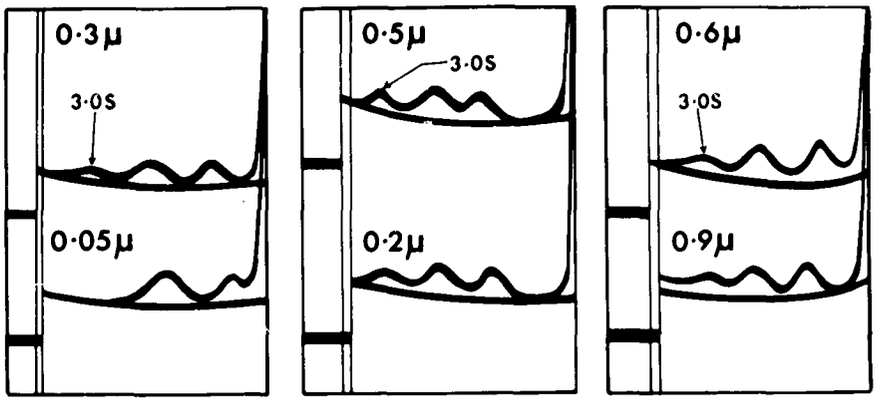


FIG. 7. Sedimentation patterns of solutions of granule proteins in solvents at pH 9.4 and ionic strength indicated. Third component only identified by S value; other components showed variability within range indicated in lower row Fig. 2.

and two components having sedimentation rates comparable with those of monomer and dimer Lv would be expected whether or not they are complexed with Pv (Fig. 2). At the lowest ionic strength (0.05) these are the only two components present but at 0.2 μ or higher, a third component, having the sedimentation coefficient of Pv, appears in all patterns. Evidently Pv occurs as a complex, either with itself or with Lv, in the granule and this complex is dissociated in solutions of high ionic strength, probably through displacement of divalent ions such as calcium. The existence of a Pv–calcium complex has been demonstrated (9) but its sedimentation rate was much higher than any component observed here. This, together with the presence of a soluble PvLv complex in phosphate buffer (4), suggests that Pv exists in this form in the granule, and was not completely dissociated from Lv under the ionic strength and other conditions used in these and earlier experiments (4).

Discussion

These results show that chromatography on TEAE-cellulose can separate the two components of lipovitellin, and also all three sedimenting components known to be present in egg yolk granules. This ion-exchanger gave essentially

complete recovery and permits a more complete description of the granule proteins or lipovitellin fraction than chromatography on Dowex-1 followed by hydroxyapatite, which gave only 60–70% recovery (3). Operative advantages of TEAE-cellulose over hydroxyapatite include availability, greater stability and reproducibility. At the ionic strength required to dissolve these proteins in the neutral region, TEAE-cellulose did not absorb the material from several phosphate-free buffers. A 0.2 *M* phosphate buffer at pH 6.8, however, had sufficient solvent power and permitted all components except β-lipovitellin to be retained by the column. Although a chromatographic method that does not retain all the components and makes use of a phosphate solution falls short of the original objectives, the procedure offered many advantages over previous methods of separation.

Complete recovery enabled the proportions of the several components to be estimated from the amount and composition of the several fractions. The granule solutions contained about 43% β-lipovitellin, 29% α-lipovitellin, 18% phosvitin, and 9% of what appears to be a lipovitellin–phosvitin complex. The ratio of α- to β-lipovitellin is therefore about 1:1.5, rather than the 1:1.8 obtained earlier (3) from ultracentrifugal measurements. The proportions and protein phosphorus contents of the fractions indicate that 18.4% of the granule proteins is phosvitin, a figure in good agreement with 17% observed earlier by ultracentrifugation (3, 4) when corrected (18%) for the Johnston-Ogston effect as described by Schachman (10).

The fractions identified as phosvitin, α- and β-lipovitellin from their chromatographic behavior have, in all important aspects, the chemical composition previously reported for these components (3, 4). Protein phosphorus is the most distinctive quantity, and this was only slightly lower for lipovitellins obtained from Dowex-1 than for those obtained by direct separation of the granule proteins on TEAE cellulose. While this suggests the presence of trace amounts of phosvitin in the latter, the phosphorus contents were well within the range of values reported for the two lipovitellins.

The lipid content of lipovitellin is evidently dependent on the preparative procedure employed and, within these limits, it cannot be regarded as a characteristic property. Preparations made by precipitation from solutions that had been extracted with ethyl ether contained about 16% lipid (11) but without ether extraction the lipid content was 18% (12) by one precipitation procedure, 20% (1) by another, and 22% or higher (3, 13) when isolated by chromatography on Dowex-1 and hydroxyapatite. The lipovitellin fractions isolated by TEAE-cellulose chromatography contained only 16% lipid although subsequent observations (14) indicate values 2–3% higher. This discrepancy may arise from some unknown bias in the techniques used for preparation or lipid estimation but there is no doubt that isolation on TEAE-cellulose yields material of lower lipid content than do earlier chromatographic procedures.

Both on Dowex-1 (4) and now on TEAE-cellulose an additional chromatographic component is eluted between the lipovitellins and phosvitin. Protein

nitrogen and phosphorus contents, sedimentation coefficients, and ultraviolet spectra all indicate that this is an interaction complex of phosvitin and lipovitellin. Using alkaline solvents (pH 9.4), it was possible to dissolve the granules and examine the effect of ionic strength on the ultracentrifugal components. At low ionic strengths there was no evidence of phosvitin as a separate component but it became evident in increasing amounts at ionic strengths above 0.2. As the sedimenting granules contain 68% (3) of the calcium in egg yolk, the simplest explanation of these results is that phosvitin and lipovitellin are complexed through divalent ions that are displaced at high ionic strengths by the univalent cations ordinarily used as solvents. Apparently the amount of complex that remains in the chromatographic solvents depends on the nature and ionic strength of the solvent.

When granules are dissolved in neutral solvents, dimer lipovitellin and phosvitin are observed as separate components (3) but this does not establish that lipovitellin is present in the granule in dimer form. The present and earlier results (4) provide presumptive evidence that the granules may contain a monomer lipovitellin–phosvitin unit and its polymers. These two proteins are present in about equimolecular proportion in terms of monomer lipovitellin (4), and phosvitin is not evident as a separate component in alkaline solvents of low ionic strength. The small amount of complex found in the neutral chromatographic solvents also had the same composition as the granules. No phosvitin was evident on ultracentrifugation but the complex did contain two components having sedimentation rates consistent with expectation for a monomer lipovitellin–phosvitin unit and its dimer. The chromatographic behavior of this complex distinguishes it from phosvitin, monomer, and dimer lipovitellin. Another distinctive feature is that the monomer unit of the complex was present in neutral solvents, whereas alkaline solvents are required to produce monomer lipovitellin in similar proportions. This evidence suggests that dimer lipovitellin may be formed following the release of phosvitin by dissolution in solvents of high ionic strength.

Acknowledgments

The authors wish to thank Mr. A. E. Castagne and Mr. D. R. Muirhead for valuable technical assistance, and Dr. W. G. Martin for helpful discussions.

References

1. F. J. Joubert and W. H. Cook. Can. J. Biochem. Physiol. **36**, 389 (1958).
2. G. Bernardi and W. H. Cook. Biochim. Biophys. Acta, **44**, 86 (1960).
3. R. W. Burley and W. H. Cook. Can. J. Biochem. Physiol. **39**, 1295 (1961).
4. M. W. Radomski and W. H. Cook. Can. J. Biochem. **42**, 395 (1964).
5. R. W. Burley and W. H. Cook. Can. J. Biochem. Physiol. **40**, 373 (1962).
6. E. A. Peterson and E. A. Chiazze. Arch. Biochem. Biophys. **99**, 136 (1962).
7. E. G. Bligh and W. J. Dyer. Can. J. Biochem. Physiol. **37**, 911 (1959).
8. W. L. Bencze and K. Schmid. Anal. Chem. **29**, 1193 (1957).
9. F. J. Joubert and W. H. Cook. Can. J. Biochem. Physiol. **36**, 399 (1958).

10. H. K. SCHACHMAN. Ultracentrifugation in biochemistry. Acad. Press Inc., New York. 1959. p. 122.
11. H. L. FEVOLD. Advan. Protein Chem. **6**, 187 (1957).
12. J. E. VANDEGAER, M. E. REICHMANN, and W. H. COOK. Arch. Biochem. Biophys. **62**, 328 (1956).
13. W. G. MARTIN, N. H. TATTRIE, and W. H. COOK. Can. J. Biochem. Physiol. **41**, 657 (1963).
14. R. A. WALLACE. Unpublished observations.
15. W. H. COOK, R. W. BURLEY, W. G. MARTIN, and J. W. HOPKINS. Biochim. Biophys. Acta, **60**, 98 (1962).